

Bone morphogenetic protein antagonist gremlin 1 is widely expressed by cancer-associated stromal cells and can promote tumor cell proliferation

Julie B. Sneddon*, Hanson H. Zhen†, Kelli Montgomery†, Matt van de Rijn†, Aaron D. Tward§, Robert West†, Hayes Gladstone†, Howard Y. Chang†, Greg S. Morganroth†, Anthony E. Oro†, and Patrick O. Brown*¶||

Departments of *Biochemistry, †Dermatology, and ‡Pathology, and ¶Howard Hughes Medical Institute, Stanford University Medical Center, Stanford, CA 94305; and §G. W. Hooper Foundation, University of California, San Francisco, CA 94143

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Although tissue microenvironments play critical roles in epithelial development and tumorigenesis, the factors mediating these effects are poorly understood. In this work, we used a genomic approach to identify factors produced by cells in the microenvironment of basal cell carcinoma (BCC) of the skin, one of the most common human cancers. The global gene expression programs of stromal cell cultures derived from human BCCs showed consistent, systematic differences from those derived from nontumor skin. The gene most consistently expressed at a higher level in BCC tumor stromal cells compared with those from nontumor skin was *GREMLIN 1*, which encodes a secreted antagonist of the bone morphogenetic protein (BMP) pathway. BMPs and their antagonists are known to play a crucial role in stem and progenitor cell biology as regulators of the balance between expansion and differentiation. Consistent with the hypothesis that BMP antagonists might have a similar role in cancer, we found *GREMLIN 1* expression in the stroma of human BCC tumors but not in normal skin *in vivo*. Furthermore, BMP 2 and 4 are expressed by BCC cells. *Ex vivo*, BMP inhibits, and Gremlin 1 promotes, proliferation of cultured BCC cells. We further found that *GREMLIN 1* is expressed by stromal cells in many carcinomas but not in the corresponding normal tissue counterparts that we examined. Our data suggest that BMP antagonists may be important constituents of tumor stroma, providing a favorable microenvironment for cancer cell survival and expansion in many cancers.

cancer biology | stem cell regulation | tissue microenvironment | tumor stroma

Tissue microenvironments play a critical role in specifying cellular niches in both the developing embryo and adult organisms (1, 2). In development, cell fate decisions are dictated not only by cell-autonomous signals but also by stimuli from the surrounding tissue microenvironment (3, 4). Similarly, in adult tissues that continue to renew throughout the lifetime of the organism, such as the skin, intestinal epithelium, and hematopoietic system, the self renewal and maturation of the stem cell population are regulated by specific molecular cues derived from the corresponding microenvironments (5–7). In the skin, hair follicle morphogenesis is regulated by signals coming from the dermal papilla, a specialized mesenchymal structure that signals to matrix stem cells located across the basement membrane (8, 9). Similarly, the modulation of stem cell activity in the intestine is also subject to cues derived from underlying mesenchymal cells that surround the crypt (10, 11). Hematopoietic stem cells are regulated in part by osteoblasts, cells that reside in the adjacent bone spicule (12, 13). In all of these cases, a crucial feature of the regulation of stem cell compartment size, location, and timing of self renewal is the production of critical factors by a specialized set of mesenchymal cells that create a customized microenvironment.

During carcinogenesis, an analogous system of specialized tissue microenvironment cells may also be important in specifying

a “tumor cell niche” that supports a self-renewing population of tumor cells. Paradoxically, although uncontrolled proliferation and survival are the cardinal characteristics of cancer cells, it can be difficult to sustain these cells away from their corresponding microenvironment, either in culture or as explants (14). There is accumulating evidence that tumor stroma influences tumor development (15, 16). Genetic studies have shown that stromal cells are altered in some inherited cancer-susceptibility syndromes (17). In breast cancer, rearrangements at several loci have been noted exclusively in tumor-associated stromal cells (18). *In vivo* and *in vitro* experiments demonstrated that human prostatic epithelial cells showed dramatic changes both in histology and growth rate when grown with human fibroblast cells derived from prostatic carcinoma, suggesting that carcinoma-derived fibroblasts can stimulate tumorigenesis (19). Others have shown that coinjection of fibroblasts with tumor epithelial cells into mice can enhance tumor formation (20).

To identify factors produced by tumor stromal cells that contribute to the initiation or maintenance of the tumor, we used a genomic approach with basal cell carcinoma (BCC) of the skin, one of the most common human neoplasms, as our model system. Previous work with human autotransplants of BCC lesions has suggested that stromal cells in the tumor tissue play a crucial role in sustaining the tumor (21). Mouse models of the disease have shown that sustained activation of the Sonic Hedgehog pathway, a major genetic component of BCC, is maintained only in the context of the animal *in vivo*; when explanted in culture, tumor cells lose pathway activity (22).

We cultured stromal cells from BCC tumor and nontumor human skin and compared those two cell populations by cDNA microarray analysis. Antagonists of the bone morphogenetic protein (BMP) pathway were among the genes most consistently and significantly differentially expressed between the two populations. Given what is already known about the role of BMPs and their antagonists in regulating stem cell compartments in normal development and physiology, we hypothesized that a similar role could be played by BMPs and BMP antagonists in the context of the tumor.

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Abbreviations: BCC, basal cell carcinoma; BMP, bone morphogenetic protein; ISH, *in situ* hybridization; IHC, immunohistochemistry.

Data deposition: The array data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE5502).

¶To whom correspondence should be addressed. E-mail: pbrown@cmgm.stanford.edu.

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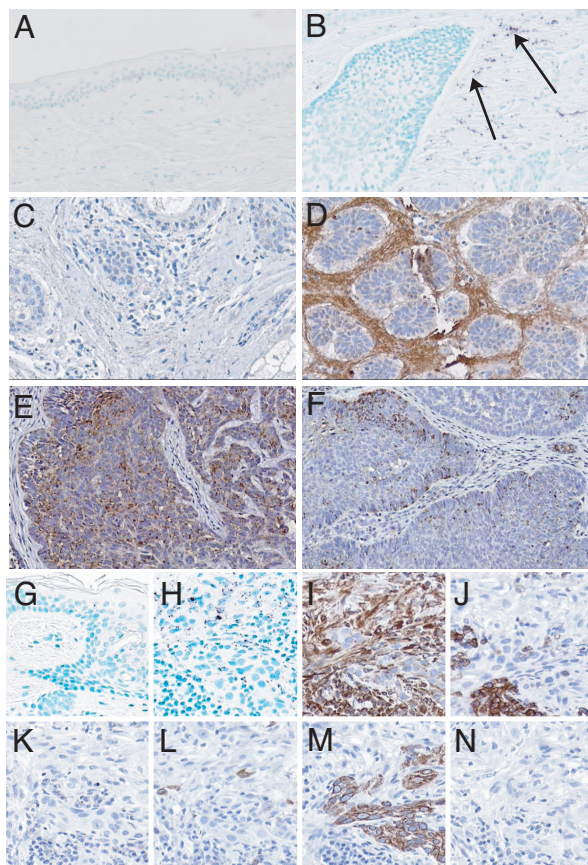


Fig. 2. Expression of *GREMLIN 1* and BMP 2 and 4 in BCC tumor tissues. (A and B) ISH for *GREMLIN 1* RNA in normal scalp (A) and BCC tumor skin (B). *GREMLIN 1* is expressed by stromal cells surrounding the tumor (indicated by arrows) but is undetectable in normal scalp. Positive signal appears as dark purple dots. (C and D) IHC for gremlin 1 protein in normal scalp (C) and BCC tumor skin (D). Positive signal appears as diffuse brown staining. (E and F) IHC with antibodies against BMP 2 (E) and 4 (F) in large sections of human BCC. Signal is represented by brown color. (G–N) serial sections of a BCC tumor showing that *GREMLIN 1*-expressing cells have properties of fibroblasts. (G and H) RNA ISH for *GREMLIN 1* RNA in nontumor (G) and tumor (H) skin. *GREMLIN 1* expression is indicated by dark purple dots. (I–N) IHC for cell lineage markers vimentin (I), CD45 (J), CD31 (K), desmin (L), pancytokeratin (M), and GFAP (N). Signal is represented by brown color.

Gremlin 1 strongly attenuated this effect. Gremlin 1 protein alone, in the absence of exogenously added BMP 4, had little effect on *SPRR* expression. [Note that basal media contains no detectable BMP (data not shown).]

Gremlin 1 also antagonized BMP-mediated repression of cell proliferation. Primary BCC keratinocytes were cultured and cell growth assessed in the presence of varying concentrations of recombinant human BMP 4 and recombinant mouse gremlin 1 (Fig. 3B). The doubling time of these cells in culture with no added BMP or gremlin 1 was 3.1 (\pm 0.1) days. Addition of gremlin 1 in the absence of added BMP 4 did not significantly affect growth rate, even at the highest concentration of gremlin (2.105 μ g/ml). In the absence of gremlin 1, doubling time increased steadily with increasing BMP 4 concentration, reaching a maximum of 7.4 (\pm 0.1) days, 2.4 times the baseline doubling rate. At the highest level of BMP 4, increasing the concentration of gremlin 1 protein steadily lowered the doubling time back to baseline. These results indicated that BMP 4 inhibits the expansion of BCC cell populations in culture, and that gremlin 1 attenuates this inhibition.

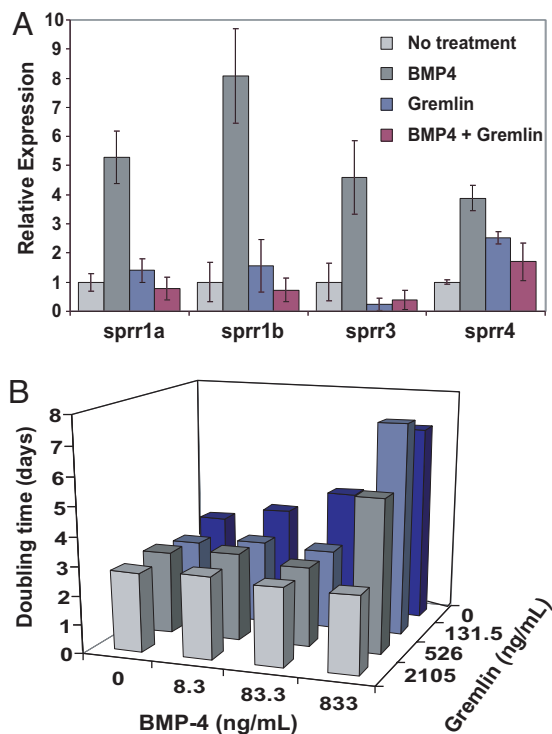


Fig. 3. Effects of BMP and gremlin 1 on BCC cell differentiation and expansion *in vitro*. (A) Cells were cultured from a human BCC tumor and then treated for 7 days in culture with recombinant human BMP 4 (833 ng/ml), recombinant mouse gremlin 1 (2,105 ng/ml), or both. Populations were then compared by using quantitative RT-PCR to detect the levels of *SPRR1A*, *SPRR1B*, *SPRR3*, and *SPRR4* transcripts. (B) Cells were cultured *in vitro* from human BCCs then treated with varying concentrations of gremlin 1 or BMP 4 protein for 7 days. Cells were counted by using a hemacytometer, with triplicate counts taken for each measurement; each measurement is the average of duplicate experiments.

***GREMLIN 1* Is Expressed by Stromal Cells in Diverse Human Carcinomas.** *GREMLIN 1* is highly expressed in the fibroblasts of most BCCs and undetectable in most normal skin sites. Evidence that BMPs regulate stem cell expansion in many tissues (skin, intestine, and blood) raised the possibility that expression of gremlin 1 may be an important feature of the tumor microenvironment in other cancers (12, 24, 25). We therefore examined *GREMLIN 1* RNA expression in a total of 774 tumors, including melanoma and carcinomas of the liver, testis, ovary, uterus, kidney, thyroid, prostate, head and neck, bladder, breast, lung, colon, pancreas, and esophagus ($n = 11$ –260 samples of each) by ISH to tissue microarrays. *GREMLIN 1* was expressed by stromal cells in at least 50% of samples in carcinoma of the bladder, breast, lung, colon, pancreas, and esophagus, and in at least 25% of prostate and head and neck cancers (Fig. 4). Expression of *GREMLIN 1* was exclusively localized to the stromal cells, with the exception of some breast and prostate samples, which showed limited expression in the tumor cells themselves.

We also examined large sections of breast, pancreas, lung, and intestine, both tumor and nontumor. *GREMLIN 1* expression was undetectable in normal and benign breast tissue. In a series of 165 samples of pancreas, including normal tissue and benign and malignant lesions, we detected *GREMLIN 1* RNA in only 5% (2/37) of normal samples, compared with 71.5% of pancreatic tumors (68/95) (Fig. 7, which is published as supporting information on the PNAS web site). *GREMLIN 1* expression was also detected in 45% (15/33) of benign pancreatic disease samples, including pancreatitis, benign neuroendocrine tumors,

counterpart, suggests this maintenance is specified by a stable genetic or epigenetic program.

The addition of gremlin 1 alone to basal media was not enough to sustain long-term culture of BCC-derived cells. Thus, future work is needed to define additional supporting factors present in the tumor cell niche. As a preliminary step, we have used RT-PCR to examine the expression of a number of other reported BMP antagonists, including *TSG1*, *FOLLISTATIN*, *NOGGIN*, and *CHORDIN*, in whole tissue samples of human BCC and matched nontumor tissue. Like *GREMLIN 1*, both *TSG1* and *CHORDIN* were typically expressed at higher levels in tumors compared with nontumor controls (Fig. 9, which is published as supporting information on the PNAS web site). Further characterization of other factors in the tumor cell niche, combined with the identification of signals derived from basal cell tumor cells, will help elucidate the reciprocal crosstalk that occurs between the tumor and its microenvironment. Along with *GREMLIN 1*, other genes that were elevated in BCC tumor-associated fibroblasts included a number of components of the Wnt signaling pathway, such as *DICKKOPF HOMOLOG 1* (*DKKI*), a secreted protein inhibitor of the Wnt signaling pathway. The Wnt proteins (along with BMPs) are targets of the Sonic Hedgehog pathway (36). In one report of Wnt pathway activity in BCC, the pattern of nuclear β -catenin showed increased staining at the periphery of tumor nests, as well as some staining in tumor-adjacent fibroblasts (37). Additional experiments will be useful in uncovering the connections between Wnt, Sonic Hedgehog, and BMP signaling in BCC.

We have shown that BMP inhibits expansion of BCC cells in culture, and that gremlin 1 can overcome this inhibition. The mechanism of gremlin 1/BMP action and downstream signaling events, however, remains unclear. Although we have not definitively addressed whether the effects of gremlin are mediated exclusively through the BMP pathway, our data on cultured cells from BCC tumors suggest that this is likely, because gremlin 1 had no appreciable effect on cell expansion unless BMP was present. Although we did observe BMP in some of the tumor cells *in vivo*, the tumor cells *in vitro* showed a response to gremlin 1 only in the presence of exogenous BMP. Thus, the level of BMP production by the cultured tumor cells was not high enough to produce a clear effect at the plated cell density, possibly because of the effects of dilution by the media or by loss of normal cell-cell interaction normally seen *in vivo*.

The expression of *GREMLIN 1* by stromal cells in diverse human carcinomas, in contrast to its rare expression in corresponding normal tissues, suggests that expression by cells in the tumor microenvironment of factors that regulate the self renewal of the tumor cells may be a general feature of human cancer. Inhibiting these critical molecular signals from the tumor microenvironment may thus be a useful therapeutic strategy. The potential parallels between stem cell-microenvironment interactions in normal development and cancer should provide fertile ground for further investigations.

Materials and Methods

Primary Human Cells. Stromal cells were isolated from discarded skin tissue from the Dermatology Clinic with approval from the Institutional Review Board (Stanford University Medical Center). Fat was removed by using a sterile scalpel and forceps; tissue was minced into ≈ 1 -mm cubes. Incubation in a six-well dish without medium at 37°C for 10 min allowed for adhesion of the tissue to the plate. Fresh media containing DMEM, 10% FBS, and penicillin-streptomycin were added, and samples were maintained at 37°C and 5% CO₂. Media were replaced every 2 days. Outgrowth of spindle-shaped cells was typically apparent after 5–15 days in culture and had a success rate of $\approx 60\%$. When the cells were near confluence, they were subcultured with 0.25%

trypsin-EDTA. Cultures were expanded until sufficient for RNA isolations (typically four passages).

Human BCC keratinocyte cultures were derived from fresh skin tissue as described (38). A small crosssectional piece of each sample was cut and fixed in 10% buffered formalin for histological confirmation. The remaining tissue was placed overnight in 5 mg/ml dispase (Gibco, Carlsbad, CA) at 4°C. The next day, epidermis was separated from dermis with dissecting forceps, minced by using sterile forceps and scalpel, and incubated in 0.05% trypsin-EDTA at 37°C for 15 min, with occasional mixing to disperse cells. After neutralization with HBSS containing 15% FBS, cells were spun down at 900 rpm in a Beckman Allegra GR centrifuge for 5 min, then resuspended in Keratinocyte serum-free media supplemented with EGF, bovine pituitary extract, and penicillin-streptomycin (Gibco). Cells were plated onto 12-well collagen I-coated plates (BD Biosciences, Franklin Lakes, NJ) and incubated at 37°C in 5% CO₂. Media were replaced every 2 days. Contamination from fibroblasts or normal keratinocytes was avoided by subjecting the culture to differential trypsinization and a transient increase in calcium concentration, respectively (39).

Microarray Procedures. Construction of human cDNA microarrays with $\approx 42,000$ elements, representing $\approx 24,000$ genes, and hybridizations was as described (40). Forty-eight hours before RNA harvest of stromal cultures, cells were washed three times in prewarmed PBS and then maintained in low serum media containing DMEM and 0.1% FBS. mRNA was harvested by using the FastTrack kit (Invitrogen, Carlsbad, CA). Universal Human Reference RNA (Stratagene, La Jolla, CA) was used as reference for array experiments.

Arrays were scanned with a GenePix 4000A scanner and images analyzed with GenePix 3.0 (Axon Instruments, Union City, CA). Microarray data were stored in the Stanford Microarray Database (41). All microarray data are available at the web site <http://microarray-pubs.stanford.edu/Gremlin1.BCC>.

Data Analysis. We considered only genes for which the cognate array element had a fluorescent signal at least 1.5-fold greater than the local background signal in both channels. Significance Analysis of Microarrays (29) was then used to identify a set of genes whose expression levels were significantly different between five tumor- and five nontumor-derived stromal cell cultures at a false discovery rate of 15% or 5%. Resulting expression patterns were organized by hierarchical clustering (42).

ISH. Digoxigenin-labeled sense and antisense riboprobes for *GREMLIN 1* were synthesized by using T7 polymerase-directed *in vitro* transcription of linearized plasmid DNA (IMAGE clone 7262108) by using the DIG RNA Labeling Kit (Roche Diagnostics). ISH on paraffin sections was performed by using a biotinyl tyramide amplification procedure, essentially as described (43). Results were considered specific when a strong pattern of distinct punctate staining was seen for the antisense probe, and little or no staining was observed for the corresponding sense probe. Tissue microarrays of tumor samples were made as described (44).

IHC. IHC staining for Gremlin 1 was performed with Dako Envision Plus (Glostrup, Denmark). Anti-gremlin 1 antibody (Imgenex, San Diego, CA) was used at 1:10 dilution. IHC for BMPs was performed by using Vectastain ELITE ABC Rabbit IgG (Vector Laboratories, Burlingame, CA). Anti-BMP 2 and 4 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used at 1:50 dilution. IHC for cell lineage specific markers was performed by using the Vectastain ELITE ABC Mouse IgG kit with antibodies against Vimentin (1:200), CD31 (1:30), CD45 (1:100), GFAP (1:100), Desmin (1:100), and pancytokeratin

